Pericytes and their potential in regenerative medicine across species

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Abstract

The discovery that pericytes are *in vivo* counterparts of Mesenchymal Stem/Stromal Cells (MSCs) has placed these perivascular cells in the research spotlight, bringing up hope for a well-characterized cell source for clinical applications, alternative to poorly-defined, heterogeneous MSCs preparations currently in use. Native pericytes express typical MSC markers and, after isolation by fluorescence-activated cell sorting, display an MSC phenotype in culture. These features have been demonstrated in different species, including humans and horses, the main targets of regenerative treatments. Significant clinical potential of pericytes has been shown by transplantation of human cells into rodent models of tissue injury, and it is hoped that future

studies will demonstrate clinical potential in veterinary species. Here, we provide an overview of the current knowledge on pericytes across different species including humans, companion and large animal models, in relation to their identification in different body tissues, methodology for prospective isolation, characterization and potential for tissue regeneration.

Key terms: Pericyte, veterinary, CD146, regenerative medicine, mesenchymal stem cells, human, horse, dog

Introduction

Pericytes were initially described as 'Rouget cells' by Eberth and Rouget (1,2) at the end of the 19th century, and were renamed by Zimmerman as 'pericytes' (3). However, other designations, although less specific, such as 'mural cells' (4-6) and 'CD146+ cells' (7,8), are also in use. Pericytes localize across the body around the endothelial layer in capillaries and microvessels, where they are embedded within the basement membrane and can be identified based on the expression of markers including CD146 (cluster of differentiation 146; MCAM, melanoma cell adhesion molecule), PDGFRβ (CD140b, platelet derived growth factor receptor beta), NG2 (neural/glial antigen 2; CSPG4, chondroitin sulfate proteoglycan 4) and αSMA (alpha smooth muscle actin; ACTA1, actin alpha 1 skeletal muscle), although these markers are not all-inclusive of the pericyte compartment. Pericytes play fundamental roles in angiogenesis and blood vessel homeostasis and function, being critical for regulation of blood vessel integrity and permeability, and blood

flow (4,9). In addition, the wide distribution of pericytes in close association with the body vasculature (Fig. 1) positions these cells at the forefront of endogenous tissue repair responses, in which they have been shown to be actively involved either directly by differentiating into specialized mesenchymal-lineage cells or indirectly by cell-to-cell interactions or release of paracrine mediators including those related to angiogenesis and immunoregulation, two fundamental processes for tissue repair and regeneration (10-12).

Interest in pericyte biology has been in the rise since their link with Mesenchymal Stem/Stromal Cells (MSCs) was identified (13-19). In parallel, the use of MSCs in regenerative medicine has been gaining momentum, as demonstrated by the number of clinical trials currently underway worldwide (658 registered trials in the NIH in USA alone) and the approval of their use already in several applications, namely, pediatric graft versus host disease (in Canada), degenerative arthritis and orthopedics (South Korea and USA), Crohn's disease (South Korea) and post-acute myocardial infarction (South Korea and India) (20,21) and ongoing clinical trials using mesoangioblasts (a pericyte subset) in dystrophic patients (22). Other than humans, interest in the clinical use of MSCs has been very significant in the horse (23-26), followed by dog (27-29) and cat (30-32). Indeed, although not always based on solid scientific and clinical evidence, a vast number of horses have been treated worldwide and continue to be treated every year for specific orthopedic conditions using different types of MSC preparations, providing a unique pre-clinical model for human therapies (23,33,34). Research in veterinary MSCs started around 20 years ago with the characterization of equine bone marrow BM-MSCs (35) followed shortly after by the first clinical studies in horses (25,26); interest in equine MSCs has since increased steadily resulting in around 400 publications at present.

In all species, MSCs for clinical use are obtained by *in vitro* expansion of relatively rare stem/progenitor cell populations in tissue extracts from bone marrow, adipose tissue or, less frequently, placenta or umbilical cord. Adipose tissue yields higher amount of MSC cells and is of easier accessibility than bone marrow, being more attractive for clinical use. Although reports indicate variation on the properties between these two tissue sources, the results did not always agree between studies, reflecting the heterogeneity between cell preparations what may also result from method of extraction, culture conditions, age and gender of donor (36). MSCs are applied either as crude cell extracts or, given their low abundance in native tissues, following expansion in culture to obtain sufficient cells for clinical use. Either way, cell preparations used clinically are heterogeneous in nature (37) and contain variable amounts of stem/progenitor cells and occasionally other contaminant cell types raising important issues regarding standardization and consistency of cell harvesting methods, and efficacy of treatments. As most of the research in this area has been performed with MSCs, it is not completely clear at the moment what cell sub-sets or combinations of these will be more appropriate for the diverse tissue regenerative applications. Systematic studies involving MSCs sub-populations are underway and more work will be needed to answer these crucial questions in order to obtain effective, reliable and consistent cell therapies (38-40). Therefore, despite guidelines from The International Society for Cellular Therapy (ISCT) defining the characteristics of culture-derived human MSC preparations (41-43), there has long been a clear interest to identify and characterize MSC sub-populations in their native form, i.e., before expansion *in vitro*. In the absence of specific criteria, ISCT guidelines are used to define MSCs from veterinary species, which does not account for species-specific differences, for example in relation to expression of cell surface antigens. In addition, because of

the lack of species-specific antibodies (34,44), cross-reactivity with MSC and pericyte markers needs to be established in each case. These challenges have hampered the identification, isolation and characterization of native MSC populations from veterinary species.

A perivascular source of MSCs

A link between MSCs and blood vessel walls was demonstrated by studies in which cells with MSC features, including expression of specific markers (CD29, CD44, CD90), multipotency and clonogenicity, were obtained from human saphena (45) and aorta (46), as well as by the demonstration that fetal mesangioblasts (a cell with pericyte characteristics) (47) and adult pericytes (48) can differentiate into distinct mesenchymal lineages. More definite proof of a perivascular origin of MSCs was provided by seminal studies showing that pericytes in different human tissues stained for classical MSC markers and, upon *in vitro* expansion, displayed an MSC phenotype while maintaining the expression of pericyte markers (CD146, PDGFRβ, NG2, αSMA). These findings have more recently extended to horse, dog and sheep (8,49-52), overall indicating that pericytes are *in vivo* counterparts of culture-expanded MSCs in different species (13,39,53). Some of the previous studies (8,54) also identified a population of adventitial cells (marked by the expression of CD34) as a perivascular cell type (in addition to pericytes) with MSC properties, and proposed these adventitial cells to be precursors of pericyte (54). However, relatively less attention has been paid to the MSC biology and regenerative potential of adventitial cells, and will not be discussed in this review.

Multipotency is a central property of MSCs and cell lineage tracking studies have shown that pericytes are indeed able to differentiate into different mesenchymal lineages *in vivo*. Thus, using

Cre-mediated PDGFRβ-reporter mouse strains, it was shown that PDGFRβ-positive cells acted as adipocyte precursors *in vitro* and when transplanted into nude mice (55), in addition to giving rise to follicular dendritic cells capable of trapping immune complexes and recruiting B cells (56). Moreover, pericytes transgenically labelled with an inducible Alkaline Phosphatase CreERT2 contributed to growth and regeneration of skeletal muscle by fusing with developing myofibers in mice, a response that was significantly increased upon injury or in chronically regenerating dystrophic muscle (48,57). A subset of pericytes expressing PDGFRβ also contributes to fibrosis, as observed in a tamoxifen-inducible Glast-CreER YFP reporter mice in which spinal cord injury resulted in recruitment of labelled cells expressing fibronectin that formed the core of the scar (58). During tooth growth and in response to injury *in vivo*, some pericytes traced by using NG2-Cre reporter mice contribute to odontoblast production, but did not account for all cell differentiation (59), although not all pericytes express NG2 (13). In contrast to these reports, a recent lineage-tracing study using an inducible Tbx18-CreERT2 mouse showed that pericytes and vascular smooth muscle cells maintained their identity during aging without significantly contributing to tissue regeneration responses by giving rise to adipocytes, myocytes or fibrosis, despite being multipotent and showing MSCs properties *in vitro* (60). These discrepancies may have resulted from the use of different lineage-tracing strategies resulting in the selection of different subset(s) of pericytes, some of which may not intrinsically act as mesenchymal progenitors (61), perhaps depending on their organ of origin and developmental stage (62). It also needs to be considered that mesenchymal precursors involved in tissue regeneration responses may include cells other than pericytes (16,59,63,64).

Prospective isolation and characterization of pericytes

The suggestion that pericytes were a native source of MSCs provided a strong drive for developing strategies to isolate and culture these cells as an alternative to the heterogeneous MSC populations used clinically. Using fluorescence-activated cells sorting (FACS), Sacchetti et al. (65) identified a small population of CD146+/CD45- cells (0.11%±0.02% of total human BM nucleated cells) that were clonogenic, displayed mural cell properties (including the expression of αSMA, NG2, calponin 1 and 3, and PDGFRβ), and, *in vivo*, were able to produce bone and organize a hematopoietic environment when transplanted to mice and to self-renew.

Concurrently, Péault's group published their classical paper identifying pericytes as progenitors of MSCs in multiple human organs and developed a methodology enabling their prospective purification using FACS (13). Specifically, because pericytes make up a relatively low fraction of cells in a tissue and, moreover, lack specific markers, their successful isolation required enzymatic digestion of tissue samples (they used adult and fetal skeletal muscle, placenta, adipose tissue, skin, myocardium, pancreas, and bone marrow) followed by FACS using a panel of antibodies to select cells expressing CD146+ after exclusion of hematopoietic (CD45), endothelial (CD34) and myogenic (CD56) populations (13). Reported abundance of human pericytes based on flow cytometry analyses ranged from <2% of total cells in skeletal muscle, pancreas or placenta to 14.6% in adipose tissue. CD146+/CD45-/CD34-/CD56- human cells expressed a variety of pericyte markers (NG2, PDGFRβ and αSMA) and gave rise in culture to cells with MSCs features, including expression of typical MSC markers, migration in a culture model of chemotaxis, classical trilineage differentiation *in vitro* at the clonal level, and myogenic and

osteogenic differentiation *in vivo* in mice. Alternative strategies to isolate pericytes have been reported including simplified versions of the antibody panel used by Crisan et al. (13) in human (7,66) and mice (67) or the inclusion of additional antibodies (48) to isolate pericytes from human skeletal muscle. As such, cells have been isolated from human heart (CD146+/CD34−/CD45−/CD56−/CD117−) (68), umbilical cord (CD146+/CD45-/CD34-/CD56-)(69), skeletal muscle (alkaline phosphatase (ALP)+/ CD56-) (48) and endometrium (CD146+/PDGFRβ+) (70), while trace amounts of cells (0.04%) natively expressing MSC markers (CD105+/CD73+/CD90+/CD34-/CD45-) have also been detected in human adipose (71). Some studies reported the use of single rather than a panel of antibodies to isolate human pericytes (66,72). Their results need to be interpreted with caution given the likelihood of inclusion of contamination by other cells (e.g. endothelial, hematopoietic and epithelial) that can significantly alter the characteristics of the populations obtained and provide equivocal data regarding clinical potential. In order to allow reliable comparison of results between studies, standardization of isolation procedures and cell culture conditions will be essential in future studies.

Pericytes for regenerative medicine

Numerous studies have investigated the potential of pericytes for human regenerative medicine primarily using rodent models (Table 1). Steps are also being made towards investigating their potential in veterinary medicine, specifically in relation to the horse, a recognized pre-clinical model for human musculoskeletal regeneration (33). Consistent with the demonstrated involvement of native mouse pericytes in endogenous tissue repair (57,58), the inclusion of CD146+/NG2+/PDGFRβ+/CD45-/CD34-/CD56- human cells in engineered vascular

grafts improved patency rates and tissue remodeling, and promoted deposition of collagen and elastin (73). In addition transplantation of CD146+/CD34-/CD45-/CD56- human cells in a SCID/NOD mouse model of myocardial infarction promoted regeneration by significantly improving contractibility, increasing host angiogenesis and reducing ventricular remodeling, myocardial fibrosis, and chronic inflammation at the infarct site. Transplanted cells secreted high levels of trophic factors and cytokines including VEGF-A, PDGF-β, TGF-β 1, IL-6, LIF, COX-2, and HMOX-1 (68). The clinical regenerative potential of human pericytes has also been tested in muscle. CD146+/CD45-/CD34-/CD56- human cells from muscle, adipose or placenta (74) were injected into either cardiotoxin-injured gastrocnemius muscle of SCID/NOD mice or gastrocnemius muscle of Duchenne muscular dystrophy (SCID/mdx) mice producing human spectrin-positive myofibers (13) and dystrophin-positive myofibers (74), in addition to promoting angiogenesis. In another study, human CD146+/CD34-/CD45-/CD31- cells obtained from lipoaspirates decreased muscle atrophy following rotator cuff tears in immunodeficient mice (75). Human CD146+ cells from umbilical cord were also studied in a murine model of arthritis, showing increased therapeutic potential compared to CD146- cells (66). Pericytes have also been tested for healing of experimental bone defects. Specifically, adipose-derived human CD146+/CD45−/CD34−/CD31− (76) and mouse CD146+/NG2+/CD45- (67) cells showed similar regeneration potential to BM-MSCs in an atrophic non-union rat model and contributed to regeneration of large bone defects in a critical size bone defect in mice, respectively.

The regenerative potential of human pericytes (CD146+/CD45−/CD34−) in combination with adventitial cells (CD146-/CD45−/CD34+) obtained simultaneously from the same stromal vascular fraction (SVF) preparations was also tested immediately after sorting in a critical-sized

calvarial bone injury model in immunodeficient mice, showing greater healing than when using unfractionated SVF (77). The combined use of the two different cell fractions in this and other studies (see below) was aimed at boosting cell numbers which, considering the relatively small numbers of each cell type normally obtained by FACS, would eventually allow transplantation of cells shortly after isolation avoiding the need for pre-expansion in culture (Fig. 2).

Regardless, the positive effects consistently observed in these studies using different tissue regeneration settings demonstrate the potential of pericytes for clinical tissue repair and regeneration. Whether pericytes have greater clinical efficacy than culture-expanded MSCs or SVF extracts is still unclear at the moment as controlled comparisons between these cell types have rarely been performed (76,77). Thus, despite important advances made to date, far more investigation is needed to fully define the potential of pericytes for regenerative medicine, particular in relation to the advantage they may provide relative to unfractionated MSC preparations used currently. In addition, their clinical potential in veterinary species has not been assessed.

Pericytes in veterinary species

Given the promising results obtained using human pericytes in animal models of tissue regeneration, significant interest has also arisen in the isolation and characterization of pericytes from veterinary species, either for the purpose of developing improved regenerative therapies for companion animals or use in pre-clinical models of human regenerative medicine. Isolation and culture of pericytes from species other than human and rodents has been particularly

challenging due to the lack of specific antibodies, and only very recently were the first successful attempts reported (in sheep, horse and dog) (8,51,52).

Horse – The widespread use of cell therapies in the horse (23,27,33,78-81) has fostered great interest in the characterization of MSCs with the ultimate aim of improving their therapeutic efficacy. Although a large number of studies have been devoted to this subject, no attempts had been reported until recently (8,50,82) to characterize different cell subpopulations with the aim of identifying subsets with optimum therapeutic properties. Earlier PCR (83) and flow cytometry (84) studies already reported the expression of the pericyte marker, CD146, in MSC preparations. More recently, we confirmed that indeed multiple pericyte markers (CD146, NG2, αSMA, PDGFRβ) are expressed by culture-expanded MSCs at least up to passage 8 (50). In addition, similar to results in humans, pericyte and MSC markers co-localized in different equine tissue as well as in cultured MSCs (8,50), overall indicating that pericytes give rise to equine MSCs in culture. Following on these results, we used a FACS procedure similar to that described for isolation of human perivascular cells (13,85-87) to separately isolate pericytes (CD146+/CD144-/CD45-/CD34-) and adventitial cells (CD146-/CD45-/CD144-/CD34+) from equine adipose SVF (8). We showed that, similar to findings in humans, native pericytes (as well as adventitial cells) could be successfully expanded in culture showing an MSC phenotype including multipotency and expression of specific MSC markers. Moreover, using chicken chorioallantoic membrane assay we showed that pericytes distinctly promoted angiogenesis *in vivo* and produce high levels of the angiogenic factors, VEGFA and ANGPT1, when compared with non-sorted SVF. Consistent with these findings, when co-cultured with endothelia, CD146+ cells became closely associated with vascular networks *in vitro*. Thus, given their ability to maintain a pericyte phenotype in culture,

especially in relation to their pro-angiogenic properties, CD146+ cells may be particularly useful for promoting tissue healing and improve the efficacy of current regenerative treatments in the horse. Further investigation is now needed to test the potential of these cells for specific applications including tendon repair, osteoarthritis, skin lesions and laminitis.

Dog – Two different groups have reported on the isolation of pericytes from canine adipose tissue using FACS. In one study, a single anti-PDGFRβ antibody was used for which cross-reactivity validation was not reported (88). PDGFRβ+ accounted for 40% of cells in the original extract, a relatively high value compared with the yield of pericytes obtained by FACS in other studies (13,70), and suggesting contamination with PDFRβ-expressing cells other than pericytes (89). Moreover the immunophenotype and multipotency of the sorted cells were not established and therefore their actual nature is unclear. Nonetheless, subsequent experiments in a canine model of stroke concluded that intra-arterial delivery of autologous PDGFRβ+ cells following hyperosmolar mannitol administration was a feasible and safe therapeutic approach. In a second study, IHC was performed to identify CD146+ cells in adipose tissue followed by isolation from SVF by FACS using a panel of antibodies, as previously described in other species (8,13,51), to isolate CD146+/CD45-/CD34- (pericytes) and CD146-/CD45-/CD34+ (adventitial cells) fractions. Characterization of each separate fraction was not reported but rather the two combined fractions were shown to be osteogenic *in vitro* and to produce trophic factors, VEGF, FGF-2 and PDGF, which expression was increased when cells were treated with the osteoinducer protein, NELL-1. The authors compared these with their previous findings with human cells and concluded that perivascular cells from dogs had less osteogenic potential than their human counterparts. Thus, although significant characterization of perivascular cells is yet to be performed in dogs,

the potential exists, as in the horse, for future clinical applications, particularly musculoskeletal disorders to which some breeds are particularly predisposed.

Sheep **–** The sheep is a recognized orthopedic research model and in that regard ovine pericytes may be particularly useful for preclinical testing of novel musculoskeletal therapies. Péault's group isolated pericytes (CD146+/CD45-/CD34-) from reportedly different ovine tissues, namely placenta, myocardium and adipose tissue, including infrapatellar fat pads (51). Unlike in some of the previous studies in humans and horse (8,87), depletion of endothelial cells was not performed due to lack of a suitable antibody, and no attempts were made to assess the level of endothelial contamination in the preparations obtained. Pericytes and adventitial cells (CD146-/CD45-/CD34+) were characterized together, and both fractions showed characteristics of MSCs including multipotency. Autologous transplantation of the two combined cell fractions, after labelling with green fluorescence protein, to a cartilage defect was followed by engraftment into the articular tissue, although no repair was observed by 4 weeks. Thus, as in horse and dog, further studies are needed to establish the actual clinical potential of ovine perivascular cells and whether this would be valuable for preclinical studies on human regenerative therapies.

Pig **–** The pig provides an extremely valuable model for degenerative conditions amenable to treatment with MSCs, most notably, cardiovascular disease. Nonetheless, in contrast to companion species and sheep, studies aimed at selectively isolating and characterizing pericytes as MSC precursors have not been reported. Several studies reported the isolation of pericytes from porcine tissues using differential cell attachment to tissue culture vessels, a non-selective method (90,91). Although pericyte markers (αSMA, PDGFRβ and NG2) were identified in these preparations, their actual pericyte content is unclear, especially when sourced from

heterogeneous tissues such as skeletal muscle (90) and, critically, their MSC and regenerative properties were not reported. Due to the increased interest in the use of the pig not only as a pre-clinical model, but also for xenotransplantation, future efforts towards the systematic isolation and characterization of perivascular cells to assess their potential for tissue repair and immunodulation are expected.

Conclusions

The identification of perivascular cells as a native source of mesenchymal progenitor cells has opened the way to the development of novel regenerative therapies with improved efficacy using well-defined cell populations. Effective protocols for FACS-based isolation and *in vitro* expansion of human pericytes have been developed and significant clinical tissue regeneration potential has been shown for these cells using animal models. Although successful isolation and culture of pericytes from other species (horses, dogs and sheep) has also been reported using similar methodologies, their potential for veterinary regenerative medicine and preclinical testing of human therapies remain largely unexplored. Critical to further progress in this area will be the validation and standardization of the procedures used to obtain cells from different species as well as the establishment of their therapeutic potential in different clinical settings, and the identification of reliable cell phenotype correlates of clinical efficacy. It is hoped that such advances will result in improved therapies not only for humans but also for companion animals.

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Figure legends

Fig. 1 Equine pericytes. (A) Immunohistochemistry of testis showing the pericyte marker NG2 (in green) surrounding the endothelial layer visualized by staining with a CD144 antibody (in red). DAPI was used to stain cell nuclei. (B) Pericytes in culture grown in DMEM with 20% FBS and 1% penicillin/streptomycin. Scale, 50 μm, is indicated by white bars. (C) Flow cytometry histograms displaying fluorescence intensity vs event counts (corresponding to the number of cells) and showing displacement of cultured pericytes (dark line) stained with CD146 (pericyte maker) and CD29, CD90 and CD105 (MSC markers) compared with the respective isotype (grey). The antibodies used for the staining were: NG2 (MAB2585; R&D Systems), CD146 (MCA2141F; BioRad), CD29 (303015; BioLegend) and CD44 (MCA1082GA; Biorad).

Fig. 2 Schematic representation showing tissue sources and cells tested for regenerative therapies.

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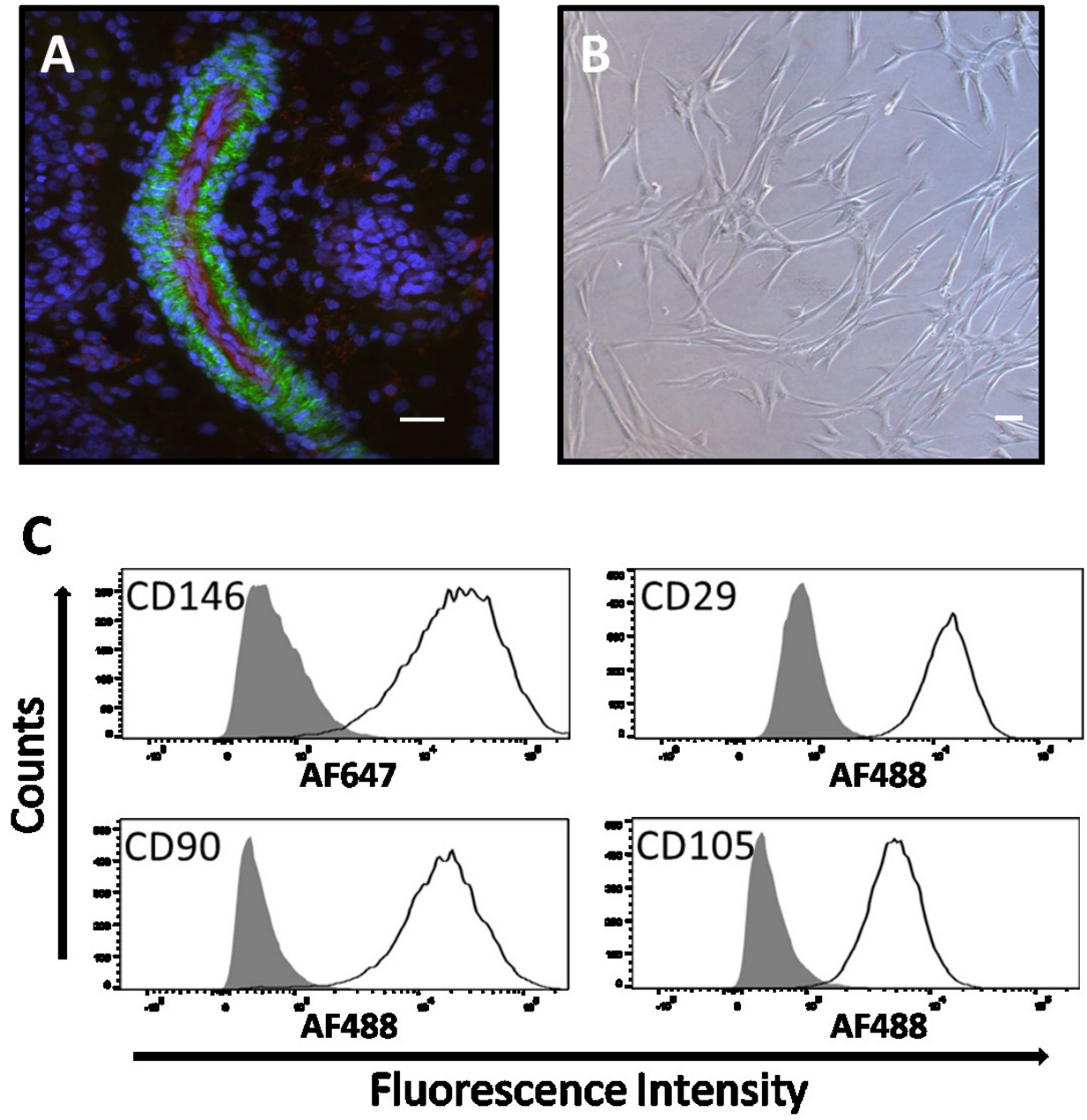
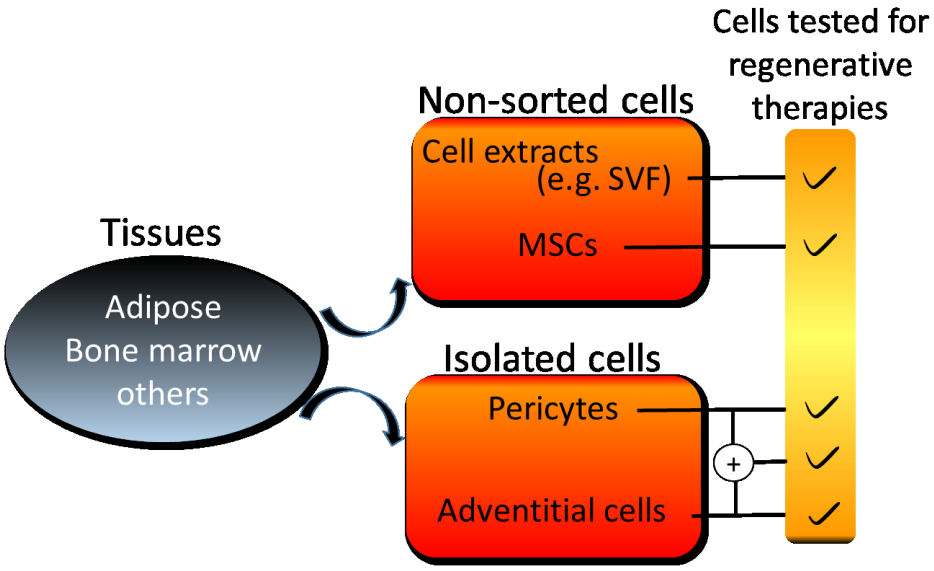
Table 1. Summary of regenerative therapy studies using isolated perivascular cells.

Tissue of origin	*Markers used for cell sorting*	*Regenerative model*
Human skeletal muscle	CD146+/NG2+/PDGFRβ+/CD45-/CD34-/CD56-	Engineered vascular grafts implanted in rat aorta {He}
Human skeletal muscle	CD146+/CD34-/CD45-/CD56-	Acute myocardial infarction in mice {Chen}
Human placenta	CD146+/CD45-/CD34-/CD56-	Cell engraftment to the gastrocnemius muscle in mice {Park}
Human lipoaspirate	CD146+/CD45-/CD34-/CD31-	Mouse model of rotator cuff tears {Eliasberg}
Human umbilical cord	CD146+	Collagen-induced arthritis in mice {Wu}
Human adipose	CD146+/CD45−/CD34−/CD31−	Rat model of atrophic non-union fracture {Tawonsawatruk}
Mouse adipose	CD146+/NG2+/CD45-	Mouse model of critical size bone defects {konig}
Human adipose	CD146+/CD45-/CD34- and CD146-/CD45-/CD34+	Critical calvalial mouse injury model {James}
Human skeletal muscle	CD146+/CD34-/CD45-/CD56-	Heart ischemia mouse model {Chen}
Human saphenous vein	CD31-/CD34+	Mouse myocardial infarction model {Katare}
Dog adipose tissue	PDGFRβ	Canine model of stroke {Youn}
Sheep infrapatellar fat	CD146+/CD45-/CD34- and CD146-/CD45-/CD34+	Sheep model of cartilage repair {Hindle}


A
B
C
CD146
AF647
CD29
AF488
CD90
AF488
CD105
AF488
Counts
Fluorescence Intensity


Cells tested for regenerative therapies
Non-sorted cells
Cell extracts (e.g. SVF)
MSCs
Tissues
Adipose
Bone marrow
others
Isolated cells
Pericytes
Adventitial cells